91-03593/

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JAPANESE SCIENCE AND TECHNOLOGY: RESULTS OF AN 8.5 MONTH STAY FELLOWSHIP AT THE FERMENTATION RESEARCH INSTITUTE, TSUKUBA, JAPAN

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Extractive Metallurgy Laboratory

February 1991

32 pp

Project: 30.46.01
Biotechnology

MINERAL SCIENCES LABORATORIES
DIVISION REPORT MSL 91-20 (TR)

MICROMEDIA

Japanese Science and Technology: Results of an 8.5 Month STA Fellowship at the Fermentation Research Institute, Tsukuba, Japan.

W. D. Gould

EXECUTIVE SUMMARY

Tsukuba, which is also referred to as Science City, was planned in the early 1960's and construction was begun several years later. The city now contains approximately 60 research institutes and two universities. The Fermentation Research Institute is located in a research park containing five other government laboratories. The largest institute in the complex, the Electrotechnical Laboratory, has over 500 scientists and the smallest, the Fermentation Research Institute (FRI) has 140 scientists. Approximately 70 of the scientific staff in the FRI are permanent government employees and 70 have been seconded from industry for periods of up to two years. However, government employees are not seconded to industry due to the excessive secrecy that is observed in Japanese industrial research. **An additional 17 are responsible for all maintenance, administrative and managerial functions. One person is responsible for all business development and technology transfer activities.** Research projects are funded for durations of three to five years and all funding, including cooperative research with industry, is provided by the government. **The Japanese do not consider cost recovery to be a viable concept.**

The FRI is divided into three departments. The **"biological function development department"** carries out research on enzyme

technology. In particular the group's research interests are in the use of enzymes in bioreactors for the production of pharmaceuticals and organic chemicals. The mandate for the **"molecular and cellular biology department"** is basic research on cellular function. They have identified and cloned genes for several important vasorepressors such as endothelin. They are also characterizing a new class of RNA molecules called ribozymes which consist of single stranded RNA with catalytic properties. The **"microbe application department"** carries out research primarily in waste treatment and environmental problems as well as some research on industrial ethanol production.

All of the laboratories within the institute are extremely well equipped with "state of the art" equipment. The budget for supplies is adequate but not as generous as the capital equipment budget. The section in which I carried out my research, "The Microbial Population Dynamics Division" has nine scientists, eight of them working on the treatment of wastes using the batch activated sludge system and one on the biological treatment of offensive odors in sewage treatment plants. Four of the scientists in the group were seconded from large companies such as Mitsubishi and Kawasaki. These companies have no biotechnology products but had formed small research groups in that field with a view towards diversifying in five to ten years. In Japan both government and industry take a much longer term view in their planning process.

During my stay in Tsukuba I attended two scientific meetings. The first was the "International Association on Water Pollution

Research and Control" in Kyoto from July 29 to August 3, 1990. The most useful symposia concerned biofilm reactors for wastewater treatment, anaerobic digestion and adsorption processes for water treatment. The symposium on biofilm reactors was particularly useful as a number of papers were presented on Rotating Biological Contactors (RBC) and fluidized bed reactors for wastewater treatment. The advantages and disadvantages of RBC's for the treatment of various effluents were discussed in detail. The main disadvantage of RBC's is that the final effluents from RBC's have a high Biological Oxygen Demand (BOD) and suspended solids content. Several of the solutions suggested for the difficulties associated with RBC's included increasing the hydraulic retention time and filtration of the final effluent.

The second meeting attended was the IUMS Congress: Bacteriology and Mycology, held in Osaka, Sept. 16-22, 1990. The most useful symposium at this meeting concerned the detection of disease-causing and genetically engineered microorganisms in the environment. The research discussed in this symposium was particularly of interest for those involved in drafting biotechnology regulations. Sensitive methods employing molecular biology techniques have been developed for the detection of both individual microorganisms and certain groups of microorganisms that occur in low numbers in the environment. However, these methods have not been sufficiently well proven to be used for regulatory purposes.

A number of contacts were made with both government and

university scientists. Contact was also made with the Metal and Mining Agency of Japan. Their environmental projects are carried out in cooperation with Dowa Mining Co. Ltd. Their main acid mine drainage treatment project is located at the Matsuo mine. The drainage from the mine contains a mixture of ferric and ferrous iron. They use a stirred tank reactor to oxidize all of the iron in part of the drainage to the ferric form and then combine the two portions. The resulting solution is then limed which causes all of the iron to precipitate as ferrite which can be removed magnetically. The treatment cost can be partially recovered by sale of the ferrite. Their treatment system would only be viable where land is at a premium and cost is not a major consideration. Thus, it is unlikely that their system could be directly applied to Canadian conditions.

My research project concerned the purification and characterization of the enzyme methyl mercaptan oxidase from Thiobacillus thioparus TK-m. Methyl sulphides such as dimethyl sulphide, dimethyl disulphide and methyl mercaptan are present in the gaseous effluents from pulp mills and sewage treatment plants. A pilot plant consisting of a packed bed column containing a culture of T. thioparus was operated for one year in a sewage treatment plant which successfully removed organic sulphur compounds from the waste gases. One of the key enzymes in the degradation pathway of these organic sulphur compounds is methyl mercaptan oxidase which catalyzes the oxidation of methyl mercaptan to formaldehyde and sulphide. Methyl mercaptan oxidase from a

Hyphomicrobium sp has been previously purified and characterized by other researchers but the enzyme from T. thioparus has not. The enzyme from T. thioparus was found to be a monomer with a molecular weight of approximately 29,000 which is smaller than the value of 50,000 that was found for the enzyme from Hyphomicrobium. The enzyme oxidized methyl mercaptan stoichiometrically to formaldehyde and sulphide. The enzyme had a Km value of 31.3 uM for methyl mercaptan. It was also shown to oxidize ethyl mercaptan. It also differed from the enzyme isolated from Hyphomicrobium in that it was not inhibited by the sulphide ion. The knowledge gained during this study will assist in the optimization of organic sulphur removal from waste gases. The purified enzyme was found to be very stable and potentially could be used in a biosensor for the detection of waste gases containing certain organic sulphur compounds.

The comments on the STA fellowship program only reflect my experiences in Japan. Other foreign researchers in Japan that I met during my stay there had varying experiences. The visiting scientists attitude and the compatibility of research interests will affect how useful and enjoyable their visit will be. My experience was exceptional as I accomplished a great deal of research and learned a lot of new biochemical techniques and gained experience in the operation of new scientific instruments. I also made a large number of new contacts and gained a great deal of general scientific knowledge. The STA program is well organized and the visiting scientists are treated very well.

INTRODUCTION

Tsukuba, which is also known as Science City, was planned in the early 1960's and construction was begun several years later. Many of the Japanese national laboratories that were located in Tokyo were moved to Tsukuba during a 20 year period. The Japanese government created the "Science City" in order to reduce crowding in Tokyo yet maintain a large research establishment in the capital region. The presence of a large number of government laboratories in one location was expected to encourage industries to locate their research laboratories in the same area. The city now contains 45 national (government) laboratories, 37 industrial laboratories and two universities.

The Fermentation Research Institute is located in a research park containing five other government laboratories, the Geological Survey of Japan and the Tsukuba office of the Agency of Industrial Science and Technology. The other government laboratories in the research park are: the Electrotechnical Laboratory (552 scientists), the National Chemical Laboratory for Industry (274 scientists), the National Research Institute of Metrology (127 scientists), the Industrial Products Research Institute (101 scientists) and the Research Institute for Polymers and Textiles (101 scientists).

The Fermentation Research Institute (FRI) which is the smallest institute in the complex, has 70 permanent government scientists and an additional 70 scientists that have been seconded from industry for periods of up to two years. However, government

employees are not seconded to industry due to the excessive secrecy that is observed in Japanese industrial research. An additional 17 employees are responsible for all maintenance, administrative and managerial functions. One person is responsible for all business development and technology transfer activities. In 1967 the FRI had 45 permanent scientists with 17 administrative employees. The laboratory now has 70 permanent scientists with the same administrative structure. The Japanese consider excess bureaucracy to be a waste of resources so they are continually streamlining administrative procedures as well as reducing the numbers of the administrative staff. The FRI functions very efficiently since little time and effort are wasted on unnecessary administrative tasks. All of the laboratories within the institute are extremely well equipped with "state of the art" equipment. The budget for supplies is adequate but not as generous as the capital equipment budget. Research projects are funded for durations of three to five years and all funding including cooperative research with industry is provided by the government. The Japanese do not consider cost recovery to be a viable concept. One of the major cooperative research project is the "Aqua-Renaissance '90 Project". The funding for this project (1985-90) was provided entirely by the government and given to a consortium that included the FRI and several companies. The project's objective was to develop new membrane-bioreactors that would produce clean effluents and methane for energy generation from wastewaters.

Technology transfer between the FRI and industry occurs very

smoothly because Japanese industry is very receptive to new technology and there are few bureaucratic obstacles to technology transfer within the FRI. The Japanese government has always provided a high level of funding for research, even during times of extreme financial restraint such as the 1950's (Figure 1). They take a much longer term view when planning their future research and technology. The FRI has generated considerable revenue from even basic research by developing patentable processes that can be licenced to industry. One example of a successful research project that has been commercialized is the procedure for the production of an enzyme called glucose oxidase which is used extensively in the food industry. The FRI over a period of time received revenue from licencing their discovery that is equivalent to two years operating budget for the institute. Licencing of technology is superior to cost recovery as a means of generating revenue.

Research Carried Out by the FRI

The FRI is divided into three departments. The three departments are divided into 13 divisions with approximately 10 staff per division. The "biological function development department" carries out research on enzyme technology. One group carries out research on the isolation of microorganisms that produce enzymes that act as acyltransferases on various polysaccharides. They are studying the relationship between enzyme structure and reactivity with a view to producing new types of acylated sugars. The knowledge gained from understanding the relationship between enzyme structure and function can be applied

to other systems where completely new chemicals could be synthesized. They are interested in developing procedures for the conversion of organometallic compounds, particularly organo-mercury compounds. Another area of research activity is the enzymatic oxidation of various organic compounds in non aqueous solvents containing low water concentrations. Non aqueous solvents alter the structures and properties of enzymes which in some cases actually increases the reaction rate by one or more orders of magnitude. In order to take advantage of the basic research in enzyme behavior, work is also being conducted into the design of bioreactors for immobilized enzymes.

The mandate for the "molecular and cellular biology department" is basic research on cellular function. One of the major achievements of this group has been the cloning and sequencing of two vasoconstrictive peptides, "endothelin" and "vasoactive intestinal contractor". The results of the research on vasoconstrictors will be of use in the understanding of certain types of heart disease. They also have a research program on the regulation of protein synthesis and the morphological development of cells. They have a research project to characterize a new group of molecules, the ribozymes, which are RNA molecules with catalytic activities similar to enzymes. They also have research projects in plant molecular biology and the production of bioactive compounds (potential pharmaceuticals) by mamalian cells.

The "microbe application department" carries out research primarily in waste treatment and environmental problems as well as

some research on industrial ethanol production. Most of the projects of interest to CANMET are carried out in this department. Although the industrial production of ethanol for use as fuel and as an industrial solvent is not yet economically feasible, they are maintaining a small research group in order to be prepared for future developments in this field. The "Aqua Rennaisance '90 Project" was one of the major research projects in this department. The development of membrane bioreactors for wastewater treatment was not completely successful but a number of significant advances were made in batch activated sludge digestion. In municipal wastewater treatment a number of chemical flocculants are used to precipitate particulates in anaerobic sludge. Microbially produced flocculants such as polysaccharides or proteins were found to be effective and environmentally acceptable. The regulation and mechanisms of biopolymer production by microorganisms is also being studied. Those studies will assist in the optimization of biopolymer production. There is potential for the use of either the microorganisms or the biopolymers as bioabsorbents. The section in which I carried out my research, "the microbial population dynamics division" has nine scientists, eight of them working on the treatment of wastes using the batch activated sludge system and one on the biological treatment of offensive odors in sewage treatment plants. Four of the scientists in the group were seconded from large companies such as Mitsubishi and Kawasaki. These companies have no biotechnology products but had formed small research groups in that field with a view towards diversifying in five to ten

years. In Japan, both government and industry take a much longer term view in their planning process.

The FRI also has a "Patent Microorganism Depository" which accepts fungi, yeasts, bacteria, actinomycetes and plant cell cultures for deposit. They do not accept pathogens or organisms that require special containment procedures (P3 or P4 level containment). The FRI has been designated as the domestic depository by the Director of the Japanese Patent Office and is also an International Depository Authority based on the Budapest Treaty.

Report: "International Association on Water Pollution Research and Control" July 29-Aug 3, 1990, Kyoto Japan.

Approximately 1300 delegates from 45 countries attended the meeting. The most useful symposia were on the use of biofilm reactors for wastewater treatment, anaerobic digestion and adsorption processes for water treatment. The symposium on biofilm reactors was particularly useful as a number of papers were presented on rotating biological contactors (RBC) and fluidized bed reactors for wastewater treatment. Trinet et al (France) studied biofilm formation in a three phase fluidized bed. The fluidized bed reactor has two advantages over the fixed bed reactor (1) fluidized bed reactors are less prone to clogging and (2) they promote rapid transfer of substrates between the liquid phase and the biomass. They found the polysaccharide content of the biofilm to increase with increased air turbulence and decreased substrate availability.

A higher polysaccharide content usually results in a more stable biofilm. Fruhen et al (Switzerland) developed a mathematical model for a flat plate biofilm reactor. They concluded that heterotrophic bacteria and nitrifying bacteria were evenly distributed throughout the biofilm. However, their conclusions were based primarily on the model and they did not have sufficient empirical data to confirm their conclusions. Suidan et al (U.S.A.) used a granular activated carbon expanded bed anaerobic reactor for the degradation of high concentrations of phenol (6000 mg/L) and acetate (11,000 mg/L) in the presence of trichloroethane (800 mg/L). They found excellent degradation (93-99%) of the total organic compounds under various operating conditions. Another approach (Heijnen et al, the Netherlands) was to use biomass immobilized on sand in an anaerobic fluidized bed reactor for wastewater treatment. They found that type of reactor to be a suitable pretreatment for industrial wastewaters. Viswanathan and Boettcher (Australia) developed a process for the removal of iron from groundwater using an aerobic fluidized bed reactor. The reactor oxidized ferrous iron to ferric iron which then precipitates as ferric hydroxide. Omura et al (Japan) used a fluidized bed reactor to oxidize ferrous iron in Acid Mine Drainage (AMD) followed by precipitation of the iron with calcium carbonate. All of the procedures for iron removal and neutralization of AMD that have been developed by the Japanese are not economically feasible for Canadian conditions. Masuda et al (Japan) reported studies on the microbial ecology of the biofilm on an RBC. They found the proportion of nitrifying, denitrifying and

heterotrophic bacteria not to change with depth in the biofilm. Thus, nitrification and denitrification occur simultaneously in the same regions of the biofilm which suggests that there are both aerobic and anaerobic microenvironments in close proximity to each other. The main advantages of the RBC are ease of operation and low operating costs. One of the major disadvantages of the RBC is that it produces an effluent with both a relatively high biological oxygen demand (BOD) and fine particle concentration. Tanaka et al (Japan) were able to reduce the BOD and fine particle concentration in the effluent from an RBC by increasing the hydraulic retention time and filtration of the final effluent. Ahn et al (South Korea) used a combined RBC settling tank which proved to be an economical method for the reduction of both suspended solids and chemical oxygen demand (COD) in the effluent.

There were several interesting papers presented in a syposium on adsorption processes. Suzuki et al (Japan) presented data on a method for using carbon fibres impregnated with zirconium oxide for the removal of phosphate from wastewater. Other techniques included the use of activated carbon for the removal of phenol from wastewater (Baudu et al, France) and chitosan for the treatment of dilute aqueous solutions of various dyes (Yoshida et al, Japan).

The symposium on anaerobic digestion concentrated primarily on the removal of sulphate from wastewater. Parkin et al, (U.S.A.) used an upflow anaerobic reactor for the removal of sulphate from industrial wastewater. Both sulphate reduction and methanogenesis occurred in the bottom one third of the reactor. They found

evidence of high concentrations of sulphide inhibiting other microbial processes. Maree et al (South Africa) used a upflow anaerobic fixed-bed reactor for the removal of sulphate from acidic groundwater. The reactor removed 90% of the sulphate and increased the pH from 3.3 to 7.0.

Report: "IUMS Congress: Bacteriology and Mycology, September 16-22, 1990, Osaka, Japan.

The most useful symposium at this meeting concerned the detection of disease-causing and genetically engineered organisms in the environment. The research discussed in this symposium was particularly of interest for those involved in drafting biotechnology regulations. In order to assess the safety of a genetically engineered microorganism that has been released into the environment it is necessary to have methods to monitor that organism in the environment. Loutit (New Zealand presented an overview of the potential and problems in the detection of microorganisms in the environment. A microorganism can be altered in some way such that the altered property will allow the organism to be detected and possibly recovered from the natural environment. The techniques to alter microorganisms in order to follow their distribution in the environment are: (1) induction of antibiotic resistance (2) addition of a gene marker by genetic engineering and (3) induction of the ability for the microorganism to utilize an unusual substrate as a sole nutrient source. Microorganisms in environmental samples can also be detected by immunoassay or by detection of diagnostic genetic sequences particular to that group

of bacteria. Senoo et al (Japan) induced a Pseudomonas paucimobilis isolate to utilize hexachlorocyclohexane as a sole carbon source. They added the organism to soil samples and were able to recover the isolate from the soil samples with high efficiencies. Shinoda (Japan) was able to identify environmental isolates of Vibrio parahaemolyticus, a severe pathogen, using an immunoassay. Winstanley et al (U.K.) added a gene marker to a strain of Pseudomonas putida in order to facilitate its recovery and identification from water samples. Aoki et al (Japan) used gene probes to identify a group of fish pathogens belonging to the genus Aeromonas. The most sensitive genetic detection technique is the polymerase chain reaction (PCR) which amplifies target DNA sequences of the microorganism of interest. Atlas et al (U.S.A.) were able to detect one bacterial cell per sample using the PCR. To date, all of the newer techniques still have procedural problems in the recovery of microorganisms from the environment, particularly from soil samples. Also, the newer techniques still are not recognized yet as being valid by the regulatory agencies. However, with improvements in techniques and additional field testing some of those techniques will be accepted in the near future.

Contacts Made

A number of contacts were made with both government and university scientists. Contact was also made with the Metal and Mining Agency of Japan. Their environmental projects are carried out in cooperation with Dowa Mining Co. Ltd. Their main acid mine

drainage treatment project is located at the Matsuo mine. The drainage from the mine contains a mixture of ferric and ferrous iron. They use a stirred tank reactor to oxidize all of the iron in part of the drainage to the ferric form and then combine the two portions. The resulting solution is then limed which causes all of the iron to precipitate as ferrite which can be removed magnetically. The treatment cost can be partially recovered by sale of the ferrite. Their treatment system would only be viable where land is at a premium and cost is not a major consideration. Thus, it is unlikely that their system could be directly applied to Canadian conditions. Dowa Mining Co. has also developed a process for the removal of hydrogen sulphide from gaseous effluents. The gas is bubbled through a reactor containing ferric sulphate which oxidizes the sulphide ions to elemental sulphur which can then be recovered. The ferric ion is reduced to the ferrous form. The ferric ions are regenerated by bacterial oxidation (Thiobacillus ferrooxidans) in a bioreactor. The ferric sulphate is then recycled back to the absorption reactor.

A list of potentially useful contacts is also included in this report. Due to differences in the culture and business practices it can be very difficult to arrange meetings with the appropriate individuals in Japanese companies. It is necessary to initially contact an individual at the right level in the appropriate department. It is also useful to have an introduction arranged by someone known to the company.

STA Research Project

My research project concerned the purification and characterization of the enzyme methyl mercaptan oxidase from Thiobacillus thioparus TK-m. Methyl sulphides such as dimethyl sulphide, dimethyl disulphide and methyl mercaptan are present in the gaseous effluents from pulp mills, oil refineries and sewage treatment plants. Since the methyl sulphides are quite toxic at higher concentrations it is difficult to grow microorganisms on these substrates. Thus, there are only a few reports in the literature on the microbial metabolism of methyl sulphides. A species of Hyphomicrobium has been shown to utilize dimethyl sulphide as a source of carbon, sulphur and energy for growth. Thiobacillus thioparus TK-m has also been shown to degrade dimethyl sulpide to carbon dioxide and sulphate. The removal of dimethyl sulphide, methyl mercaptan and hydrogen sulphide from gas using a laboratory scale packed column containing immobilized T. thioparus TK-m has been demonstrated. This system has been scaled up to a pilot plant and installed in a sewage treatment facility. The pilot plant waste gas treatment system performed very well for the duration of the one year test period. One of the key enzymes in the degradation pathway of these organic sulphur compounds is methyl mercaptan oxidase which catalyses the oxidation of methyl mercaptan to formaldehyde and sulphide. By understanding the properties of this enzyme and factors regulating its activity, it may be possible to improve the efficiency of biological deodorizing systems. The knowledge gained during this study will assist in the optimization

of organic sulphur removal from waste gases. The purified enzyme was found to be very stable and potentially could be used in a biosensor for the detection of waste gases containing certain organic sulphur compounds. The details of the research are listed in the attached report.

General Comments on the STA Fellowship

The comments on the STA fellowship program only reflect my experiences in Japan. Other foreign researchers in Japan that I met during my stay there had varying experiences. The visiting scientists attitude and the compatibility of research interests will affect how useful and enjoyable their visit will be. My experience was exceptional as I accomplished a great deal of research and learned a lot of new biochemical techniques and gained experience in the operation of new scientific instruments. I also made a large number of new contacts and gained a great deal of general scientific knowledge. The STA program is well organized and the visiting scientists are treated very well.

List of Contacts

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Phone: 0298-54-6026
Research interests: degradation of organic sulphur compounds and deodorization of malodorous gases. Dr. Kanagawa was my principle contact and colleague during my stay in Japan.

Dr. Y. Kamagata
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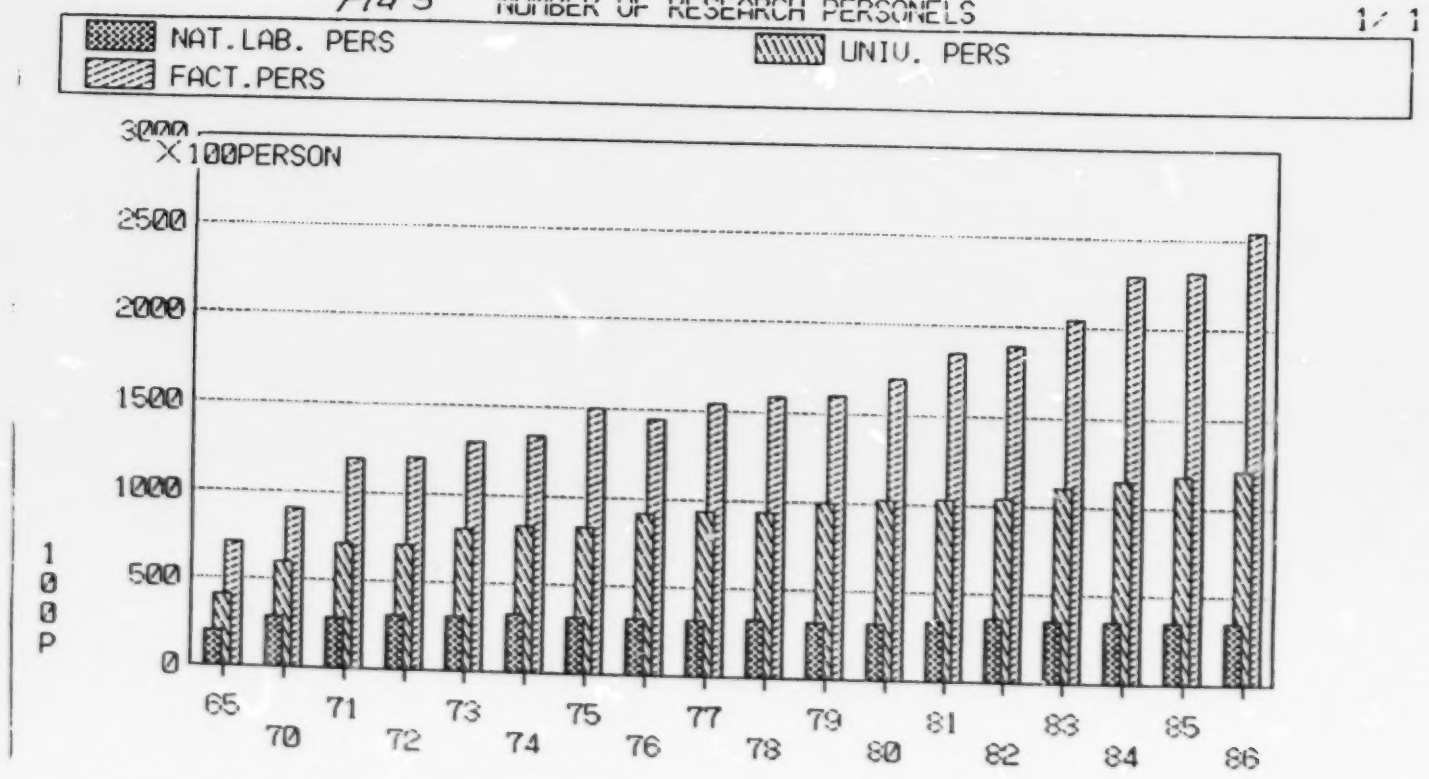
Research interests: degradation of thiocyanate by Thiobacillus thioparus

FIG 3 NUMBER OF RESEARCH PERSONELS



PURIFICATION AND PROPERTIES OF METHYL MERCAPTAN OXIDASE FROM *THIOBACILLUS THIOPARUS* TK-m

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Running title: Methyl mercaptan oxidase

SUMMARY

Methyl mercaptan (MM)-oxidase was purified to near homogeneity from *Thiobacillus thioparus* TK-m grown on dimethyl sulphide. The enzyme was a monomer with an Mr value of approximately 29,000. It oxidized MM stoichiometrically to formaldehyde and sulphide. The enzyme had a Km of 31.3 uM for MM. It was also shown to oxidize ethyl mercaptan. MM-oxidase from *T. thioparus* was shown to have a number of different characteristics from MM-oxidase of *Hyphomicrobium* EG such as a lower Mr value, higher Km and the absence of inhibition by the sulphide ion.

INTRODUCTION

Methyl sulphides such as dimethyl sulphide (DMS), dimethyl disulphide (DMDS) and methyl mercaptan (MM) are among the major volatile organic sulphur compounds produced in nature (Andreae and Raemdonck, 1983; Bremner and Steele, 1978; Lovelock *et al*, 1972; Nguyen *et al*, 1978). Several strains of *Hyphomicrobium* (de Bont *et al*, 1981; Suylen and Kuenen, 1986; Suylen *et al*, 1986) and *Thiobacillus thioparus* TK-m (Kanagawa and Kelly, 1986; Kanagawa and Mikami, 1989) have been shown to metabolize methyl sulphides. *T. thioparus* TK-m oxidized MM, DMS, DMDS and hydrogen sulphide in contaminated air stoichiometrically to sulphate (Kanagawa and Mikami, 1989; Tanji *et al*, 1989). De Bont *et al* (1981) isolated a *Hyphomicrobium* sp capable of growth on dimethyl sulphoxide (DMSO) and DMS. They suggested that DMSO metabolism involves an initial reduction of the substrate to DMS which is subsequently oxidized to formaldehyde and MM. MM is further oxidized to formaldehyde and the sulphide ion.

One of the key enzymes in this pathway is MM-oxidase which catalyzes the oxidation of methyl mercaptan to formaldehyde and sulphide with the production of H_2O_2 (Suylen *et al*, 1987). MM-oxidase from *Hyphomicrobium* EG was purified and characterized by Suylen *et al* (1987). Ethyl mercaptan and sulphide were also oxidized by the enzyme, although sulphide was a non-competitive inhibitor for MM oxidation.

The objectives of this study were to purify and characterize MM-oxidase from *T. thioparus* TK-m.

METHODS

Culture Conditions

The cells of *T. thioparus* TK-m were obtained from a continuous culture column in which DMS was the sole energy source. The inoculum for the column was grown in 100 ml of medium C (Kanagawa and Kelly, 1986) containing 2 mM $Na_2S_2O_3$ in a 500 mL Erlenmeyer flask. The flask was placed on a rotary shaker at 25°C and sealed with a gum stopper. At one day intervals for two days 5ul of dimethyl sulphide were added. After an additional 24h this culture was added to 1100 ml of medium C containing 2 mM of $Na_2S_2O_3$ which also contained trace metal solution (4ml/l) and 1.8 g/l of ammonium chloride. The medium was then added to a continuous culture column (60 mm by 570

mm) which was run for three days at 25°C using $Na_2S_2O_3$ as a substrate. DMS gas was introduced into the column and the concentration of DMS was increased as the cells grew. After one week continuous culture ($d = 0.028h^{-1}$) was begun and this was carried on for 25 days. The culture was carried out under aseptic conditions. At regular intervals the cells were harvested, washed once with 26 mM potassium phosphate buffer (pH 6.8) and stored at -80°C.

Chemicals

Reagents for protein determination and protein standards for molecular weight determination were all obtained from Sigma Chemical Co. DEAE-Sephacel was obtained from Pharmacia LKB, Biogel P60 from Bio-Rad Laboratories and SDS-Page geis were obtained from Daiichi Pure Chemicals Limited. N-ethyl maleimide (NEM) was obtained from Wako Chemical Co. ltd. All other chemicals were of reagent grade.

Purification of MM Oxidase

Approximately 1.2 g (dry weight) of *T. thioparus* TK-m cells were washed with 50 ml of 50 mM TRIS/HCl pH 8.2 buffer and then centrifuged for 30 minutes at 20,000 x g. The cells were suspended in 30 ml of 10 mM TRIS/HCl pH8.2 buffer and sonicated five times for 30 seconds at full power (200W Tomy UD201). The cell suspension was made up to 60 ml with 10 mM TRIS/HCl pH 8.2 buffer and calcium chloride was added to a final concentration of 2.0 mM. The calcium chloride was added to floculate and precipitate collodial material. The cell suspension was centrifuged at 20,000 x g for 40 minutes.

The cell-free extract was made up to 100 ml in 10 mM TRIS/HCl pH 8.2 containing a final concentration of 40 mM KCl. The extract was then eluted through a column (20 x 2.5 cm) containing DEAE-Sephacel. The column was then washed with 100 ml of the elution buffer. The eluate plus washing buffer were combined and eluted through a second DEAE-Sephacel column of the same dimensions. The second column was also washed with 100 ml of the elution buffer. The eluate plus washing buffer were combined and concentrated using an Amicon ultrafiltration unit with a PM10 membrane filter. The eluate was concentrated tenfold and made up to 200 ml with 10 mM TRIS/HCl pH8.2 buffer. This was repeated once in order to remove residual KCl and the partially purified extract was concentrated to approximately 10 ml for further purification. Three ml of the partially purified extract were saved for analysis and 7.0 ml were

applied to a gel (Bio-Rad P60) filtration column (100 x 2.6 cm) and eluted with 50 mM TRIS/HCl pH 8.2 containing 100 mM KCl at a flow rate of 8.0 ml/h.

Mr and Purity Check

The Mr of the enzyme was determined by gel permeation chromatography (Bio-Rad P60 gel) and FPLC. FPLC was carried out using 50 mM TRIS/HCl buffer, pH 8.2 containing 200 mM KCl at a flow rate of 0.5 mL/minute and a Pharmacia Superose ^{TM} 12 column. The subunit Mr was determined by SDS-PAGE (Laemmli, 1970).

Enzyme Assays

The substrate was prepared by first bubbling N_2 gas through 10 mM $P0_4$ buffer pH7.5 in a sealed vial for 0.5h. Then methyl MM (5 ml/l in N_2) was bubbled through the same buffer for 3.0 h. The MM concentration was determined using a total organic carbon (TOC) analyzer (TOC-500; Shimadzu). MM concentrations between 1.3 to 1.5 mM were consistently obtained by this method. Enzyme assays were performed in duplicate by determination of the initial product of MM oxidation, formaldehyde. A 30 second assay time was used, the reaction was stopped by the addition of an equal volume of 1.3% perchloric acid and formaldehyde was determined colorimetrically (Nash, 1953). All enzyme assays were carried out in 50 mM TRIS/HCl pH 8.2 buffer with an initial substrate concentration of 75 uM. The activity of MM-oxidase towards several other substrates was also measured using an oxygen electrode (Oxygraph-8; Central Kagaku Co., Ltd.). The assay was carried out at 30°C in a 2.8 ml cell and the reaction was initiated by the addition of enzyme. Oxidation of sulphide by MM-oxidase was determined spectrophotometrically by measuring the decrease in absorbance at 254nm. The effect of pH on MM-oxidase activity was determined using 25 mM citric acid-sodium phosphate buffer over th pH range of 3.0 to 8.0 and 25 mM glycine-sodium hydroxide buffer over the range of 8.6 to 10.6.

Inhibition of MM-oxidase

Various inhibitors were preincubated for 20 minutes with MM-oxidase, followed by determination of MM-oxidase activity by measurement of formaldehyde production. The enzyme assay was carried out using 60 ug of enzyme in 2.0 ml reaction mixture at a temperature of 25°C, a substrate concentration of 75 uM and a time duration of 2.0 minutes.

Other Procedures

Protein was determined by the Lowry procedure (Lowry *et al*, 1951).

RESULTS

A typical purification procedure is shown in Table 1. The enzyme coeluted with carbonic anhydrase on the Biogel P60 column indicating an Mr value of approximately 29,000. This result was also confirmed by chromatography on FPLC. On SDS-PAGE the enzyme proved to be nearly homogenous with a subunit Mr of approximately 29,000.

Maximum enzyme activity was observed between the pH values of 8.0 and 9.6. The Km of MM-oxidase at pH 8.2 was calculated to be 31.3 uM and substrate inhibition of the enzyme at high MM concentrations was observed (Figure 1). Oxygen consumption occurred in the presence of both methyl mercaptan and ethyl mercaptan but no activity was observed when either methyl amine or methanol were used as substrates. Oxygen consumption equivalent to 90% of the added MM occurred after the enzyme had been incubated for with the substrate for 10 minutes. The data for the oxidation of sulphide by MM-oxidase were highly variable and the role of sulphide as a substrate for the enzyme is yet to be determined.

Methyl amine and sulphide had no effect on MM-oxidase activity (Table 2). However, mercaptoethanol, ethyl mercaptan, cyanide and ammonium salts were shown to inhibit the enzyme. MM-oxidase was also inhibited by NEM, but with the exception of Cu^{+2}, metal ions had no effect on the enzyme.

When T. thioparus TK-m was grown on Na2S203 as the sole energy source, the cells had no MM-oxidase activity and the protein band on SDS-PAGE corresponding to an Mr value of 29,000 was not observed. Both MM-oxidase activity and the corresponding protein band on SDS-PAGE were observed when *T. thioparus* TK-m was grown on DMS.

DISCUSSION

MM-oxidase readily elutes through the DEAE-Sephacel column at pH 8.2 which results in an effective and convenient initial purification step that also provides a high yield of the enzyme. The Mr of MM-oxidase was identical under denaturing and native conditions which indicates that the enzyme is a

monomer. The Mr value of MM-oxidase from *T. thioparus* is significantly lower than the value of 40000-50000 reported for MM-oxidase from *Hyphomicrobium* EG (Suylen et al, 1987). Another major difference between the enzymes obtained from the two different sources is that MM-oxidase from *T. thioparus* did not bind to DEAE-Sephacel whereas the enzyme from *Hyphomicrobium* did. This suggests that there are significant differences in the surface charges of the two enzymes.

The Km value for MM-oxidase from *T. thioparus* is significantly higher higher than that of the enzyme from *Hyphomicrobium* EG. However, the enzyme from both sources appears to be inhibited by high substrate concentrations. Oxygen uptake experiments indicate that MM-oxidase from *T. thioparus* is specific for mercaptans with no activity towards amines or alcohols which has also been observed for the enzyme from *Hyphomicrobium* sp.

The differences in the magnitude of inhibition by various inhibitors on MM-oxidase from the two sources suggests that there may be differences in the structure of the active site between the two enzymes. Another major difference between the enzyme from the two different sources is the lack of inhibition by one of the main products, sulphide. The lack of inhibition by sulphide is also consistent with the fact that the enzyme from *T. thioparus* completely oxidizes MM whereas approximately 50% of the added substrate is oxidized by the enzyme from *Hyphomicrobium*. The inhibition of enzyme activity by NEM is consistent with the presence of a sulfhydryl group at the active site, however the lack of inhibition by most of the metal ions tested is not consistent with the involvement of a sulfhydryl group in catalysis. NEM has also been shown to react with a-amino functional groups (Plapp, 1983). Thus, our studies have also not yet provided definitive evidence for the prosthetic groups of MM-oxidase.

The absence of MM oxidase activity when *T. thioparus* was grown on $Na_2S_2O_3$ implies that the enzyme is inducible.

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TABLE 1

Purification of methyl mercaptan oxidase.

MM-oxidase activity was determined at a final concentration of 75uM-MM, using the formaldehyde assay.

	Volume (mL)	Total units (umol/min)	Total protein (mg)	Specific activity umol/min /mg protein	Purification ratio	Yield %
Crude extract	60	38.9	794	0.0490	1	100
DEAE-Sephacel	10	52.7	39.8	1.32	26.9	135
Gel filtration	21	54.0	17.0	3.17	64.7	139

TABLE 2

Sensitivity of MM-oxidase from *T. thioparus* towards various known enzyme inhibitors.

Compound	Inhibition	Concentration	% Inhibition at the threshold concentration
Mercaptoethanol	+	0.5 mM	53
Ethyl mercaptan	+	1.7 mM	75
Methyl amine	-	1.0 mM	
DMS	-	1.0 mM	
CH_3OH	-	100 mM	
S^{2-}	-	1.0 mM	
$Na_2S_2O_3$	-	5 mM	
$(NH_4)_2SO_4$	+	10 mM	53
NH_4Cl	+	10 mM	53
K_2SO_4	-	50 mM	
KCN	+	0.5 mM	58
EDTA	-	1.0 mM	
NEM	+	100 uM	64
$CuSO_4$	+	100 uM	55
$CdCl_2$	-	1.0 mM	
$ZnSO_4$	-	1.0 mM	
$NiSO_4$	-	1.0 mM	

* Threshold concentration at which inhibition (>50%) was observed or maximum concentration at which no inhibition was observed.